B12

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 33/86 // A61K 9/127

A1

(11) International Publication Number: WO 93/07492

(43) International Publication Date: 15 April 1993 (15.04.93)

(21) International Application Number: PCT/US92/08281

(22) International Filing Date: 25 September 1992 (25.09.92)

(30) Priority data:
771,294 4 October 1991 (04.10.91) US

(71) Applicant: BAXTER DIAGNOSTICS INC. [US/US]; One Baxter Parkway, Deerfield, IL 60015 (US).

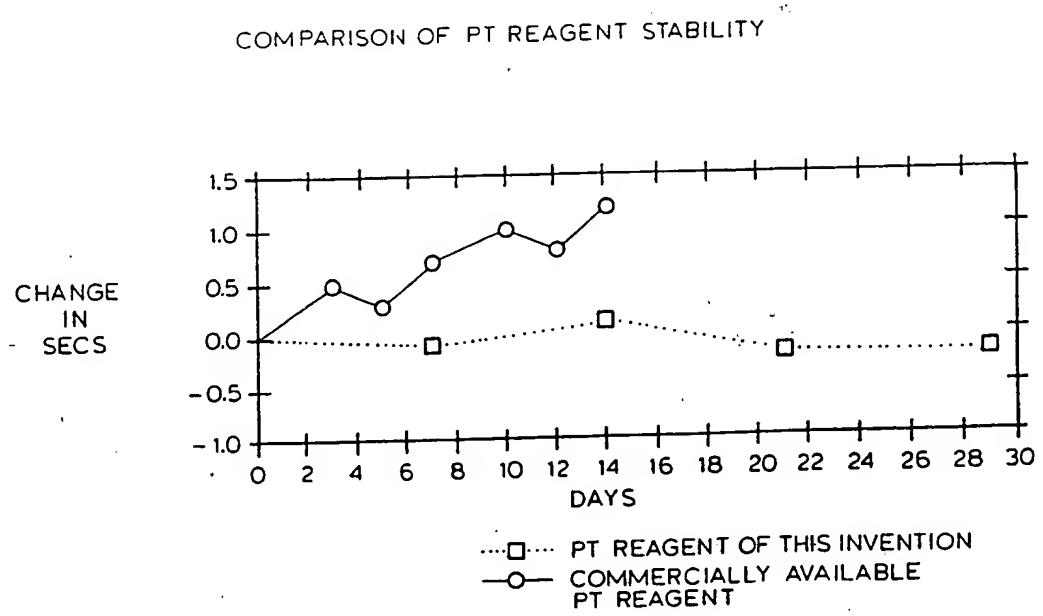
(72) Inventors: HAWKINS, Pamela, L. ; 8262 N.W. 198th Street, Hialeah, FL 33015 (US). TEJIDOR, Liliana ; 4349 S.W. 148th Avenue Ct., Miami, FL 33185 (US). MAYNARD, James ; 3104 L High Glen Drive, Charlotte, NC 28269 (US). JOHNSON, Kevin, B. ; 6601 SW 116 Court, #108, Miami, FL 33173 (US).

(74) Agents: PEARSON, Louise, S. et al.; One Baxter Parkway, Deerfield, IL 60015 (US).

(81) Designated States: AU, CA, JP, European patent (AT. BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL. SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PREPARATION OF PROTHROMBIN TIME REAGENTS FROM RECOMBINANT HUMAN TISSUE FACTOR AND PURIFIED NATURAL AND SYNTHETIC PHOSPHOLIPIDS



(57) Abstract

A prothrombin time reagent is disclosed for use in a prothrombin time test. The reagent utilizes recombinant human tissue factor, phospholipids of a natural or synthetic origin, a buffer composition and calcium ion. Stabilizers and salts may also be utilized in the reagent. In addition, a method for creating lipid micelles containing tissue factor is also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SK	Slovak Republic
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
UA	Ukraine
US	United States of America
VN	Viet Nam

Preparation of Prothrombin Time Reagents from Recombinant Human Tissue Factor and Purified Natural and Synthetic Phospholipids

Field of the Invention

The present invention relates generally to the field of Prothrombin Time reagents for determining dysfunction in the coagulation system and more specifically to reagents made from recombinant human tissue factor and phospholipids from a natural or synthetic source for Prothrombin Time tests. The present invention also includes a method to combine tissue factor with phospholipids.

Description of the Prior Art

Tissue factor, also called thromboplastin, is a membrane-associated glycoprotein which functions by forming a complex with blood coagulation factors VII and VIIa. The Complexing of these factors greatly enhances the proteolytic activity of factors VII and VIIa. Functional activity of tissue factor has an absolute dependence on the presence of phospholipids. Bach, Ronald R., Initiation of Coagulation by Tissue Factor, CRC Critical Reviews in Biochemistry 1988; 23 (4):pp.339-368. The factor VII/VIIa/tissue factor complex activates a series of specific enzymes that comprise the extrinsic and common pathways of the coagulation cascades ultimately leading to the formation of thrombin, fibrin, platelet activation, and finally clot formation. Nemerson, Yale, Tissue Factor and Hemostasis, Blood 1988; 71:pp.1-8.

Diagnostic tests such as the Prothrombin Time (PT) test, utilize this series of enzymatic events in vitro under controlled conditions to diagnose dysfunctions in the blood coagulation system of patients. In the PT

test, the time it takes for clot formation to occur is the Prothrombin time of PT value.

All currently available PT tests utilize a PT reagent containing crude tissue factor extracted from natural sources. It is important for a PT reagent to have the following characteristics: sensitive to abnormal samples, a well defined normal PT value for normal plasma, give accurate and reproducible results, have lot-to-lot consistency, must be stable for storage in the freeze-dried (lyophilized) state and must be stable after reconstitution.

Currently, the tissue factor used in the PT reagents is a crude tissue factor preparation extracted from rabbit brain, rabbit brain/lung mixtures, human placenta or ox brain. Each of these sources has limitations that make them problematic. For example, rabbit brain thromboplastin shows some seasonal variability, lot-to-lot variability and is in relatively short supply. Human tissue factor may be a source of HIV or other human viral diseases, and ox brain gives normal PT values that are much longer than those observed using tissue factor from the other common sources. Longer PT values lead to less throughput in the laboratory. Additionally, these longer times may reflect differences in the ability of ox tissue factor to bind human factor VII. Moreover, crude tissue factor preparations from natural sources contain other coagulation factors as contaminants. Contamination with coagulation factors results in coagulation factor assay curves that are less sensitive to coagulation factor-deficient plasmas. Therefore, a source of tissue factor which does not suffer from these drawbacks and has improved lot-to-lot variability is required to create a mor reproducible PT reagent. Recently, use of

recombinant tissue factor has been suggested for use in the currently available PT tests. Pabrosky, L. et al, Purification of Recombinant Human Tissue Factor, Biochemistry 1989; 28 (20):pp.8072-8077.

In the present invention, human tissue factor, which has just recently been cloned and expressed in several types of organisms including E. coli, is used in the PT reagent to solve these problems. Konigsberg W.H., Nemerson, Y. et al. Isolation of cDNA clones coding for human tissue factor: Primary structure of the protein and cDNA, Proc. Natl. Acad. Sci. 1987; 84:pp.5148-5152. In addition, the present invention can use a portion of the cloned tissue factor in the PT reagent. For example most of the intracellular (cytoplasmic) domain of the cloned tissue factor can be truncated without loss of functional activity. Further, point mutations, such as the conversion of Cys 245 to serine can be accomplished without loss of functional activity. Pabrosky L., et al., Purification of Recombinant Human Tissue Factor, Biochemistry 1989: 28 (20) pp.8072-8077.

As previously mentioned, tissue factor has an absolute requirement for phospholipids for functional activity. The phospholipids currently found in PT reagents are however, those lipids that adhere to tissue factor when it is extracted from animal sources. For example, the extraction process of rabbit brain results in the concurrent isolation of both tissue factor and naturally occurring phospholipids which are bound to the tissue factor in vivo and survive the extraction process. No further lipids are added. Therefore the nature, quantity and quality of the lipids used in the PT reagent will vary depending on the starting tissues and the extraction process. This variation may add to

lot-to-lot inconsistencies in PT reagents. The Dade^{R} thromboplastin reagents, Thromboplastin C, C+, and IS, are all based on extracts of acetone-dehydrated mixtures of buffers and stabilizers. The partially purified tissue factor extract is not completely delipidized therefore lipids are not added back into the extract, and the nature and composition of the lipids are poorly defined and variable from lot-to-lot.

In vitro tissue factor studies have shown phosphatidyl serine: phosphatidyl choline in the range of 20:80 to 40:60 restore the activity of apo-tissue factor. Nemerson, Yale, Tissue Factor and Hemostasis, Blood 1988; 71:pp.108. The nature of the polar head group on the phospholipid dramatically alters the activity of tissue factor. Bach, Ronald, Initiation of Coagulation by Tissue Factor, CRC Critical Reviews in Biochemistry 1988; 23 (4): pp.339-368. Generally, however, the phospholipids used in PT reagents have not been well characterized. A well defined and reproducible composition of phospholipids is needed to provide an improved PT reagent.

Summary of the Invention

This invention relates to PT reagents prepared using purified recombinant human tissue factor (rTF). This invention also describes the use of highly purified well defined lipids, either synthetic or natural in combination with the recombinant or native tissue factor. By controlling the tissue factor source and purity and using highly purified lipids in conjunction with well defined specific buffers and stabilizers, control of the performance of tissue factor in a PT reagent is improved.

The present invention is a PT reagent that comprises the following: recombinant tissue factor, phospholipids, either synthetic or natural, calcium ion, and a buffer composition and may also have stabilizers such as glycine or dextrans, and salts such as NaCl.

The preferred embodiment of the present invention uses a recombinant tissue factor or portion thereof obtained by the methods of Nemerson, Y. et al. Isolation of cDNA clones coding for human tissue factor: Primary structure of the protein and cDNA, Proc. Natl. Acad. Sci. 1987; 84:pp.5148-5152. Pabrosky L., et al, Purification of Recombinant Human Tissue Factor, Biochemistry 1989: 28 (20) pp.8072-8077. The preferred embodiment of the invention comprises rTF, which may be truncated or contain point mutations which have comparable activity, at a concentration of about 20 to 400 ng/mL, a phospholipid composition containing, preferably, either purified natural or synthetic phosphatidyl serine:phosphatidyl choline and synthetic derivatives thereof in the ratios of about 30:70 and having a tissue factor:phospholipid molar ratio of about 1:2000 to 1:20,000, and buffers and stabilizers. The buffers of the preferred embodiment are selected from the group consisting of HEPES, TAPSO, MOPS, TES, DIPSO, POPSO, and TRIS in a concentration of about 20 to 80 mM, however other buffers may be used. The bulking agents of the preferred embodiment include glycine in the range of about 0-10% and dextran from about 0-5%, however other agents may be used. The preferred embodiments also contain from about 9 to 15 mM calcium ion and may include about 0 to 300 mM NaCl.

The present invention also comprises a method to combine tissue factor with phospholipids. The phospholipids are solubilized in a detergent with a

critical micelle concentration high enough to allow its dialysis or diafiltration. The tissue factor is also dissolved in a detergent and combined with the phospholipids. The mixture then undergoes diafiltration in a tangential flow system, making contact with the exterior of the membrane. The diafiltration is continued until essentially all the detergent is removed.

In accordance with this invention, a PT reagent is provided which has a high degree of sensitivity and reproducibility for determining PT values. A further object of this invention is to provide a PT reagent which is sensitive to the overall function of the coagulation system. Another object of this invention is to provide a PT reagent with a well-defined clotting time for normal plasma samples and which prolongs the clotting times of abnormal plasma samples. It is a further object of this invention to provide a PT reagent with minimal lot-to-lot variability and enhanced stability and optical clarity. It is a further object of this invention to provide a method to combine the rTF with the phospholipids which is more efficient and reproducible than current methods.

The advantages and composition of the present invention will be better understood by reference to the following detailed description and examples.

Brief Description of the Drawings

Figure 1 is a graph showing the improvement in stability of a reconstituted lyophilized PT reagent of this invention over a commercially available reagent.

Figure 2 is a graph showing the improvement in stability of a lyophilized PT reagent of this invention over a commercially available reagent.

Detailed Description of the Preferred Embodiment

The advantage that the preferred embodiment of the present invention has over the prior art is that it uses a well defined, purified rTF protein in combination with purified, well defined phospholipids. Full length as well as truncated recombinant molecules can be used pursuant to the methods of Nemerson and Pabrosky. The present invention also encompasses a rTF with additions, deletions and substitutions of amino acids that do not diminish the functional activity of the PT reagent. The preferred modification of rTF is truncated at or about amino acid residue 243. The preferred concentrations of rTF in the PT reagent are from about 20 to 400 ng/mL and most preferably about 40 to 250 ng/mL. PT reagents made from these raw materials are optically clear without the fine precipitates found in PT reagents based on crude extracts of natural source materials. Since the raw materials are highly purified, chemical analysis gives a meaningful measure of their expected performance. Chemical analysis, in combination with functional assays, help provide lot-to-lot consistency, an important clinical consideration. Table I shows a comparison of three different lots of a PT reagent made using rTF. Results demonstrate the consistency of the lots by comparing PT values from a normal plasma, a normal control, an abnormal control and a warfarinized sample.

LOT TO LOT REPRODUCIBILITY

Lot No.	Normal Plasma	Warfarin Plasma	Normal Control	Abnormal Control
1	11.5	33.9	11.3	28.1
2	11.8	32.4	11.4	25.9
3	12.0	30.4	11.2	30.4

TABLE I

Naturally occuring phospholipids used in the PT reagent containing recombinant TF include natural phosphatidyl serine (PS) in the range from about 25 to 35% of total phospholipid with the most preferred at about 30% and natural phosphatidyl choline (PC) in the range from about 65 to 75% of total phospholipid with the most preferred at about 70%. The phosphatidyl choline used is neutral in charge, while the phosphatidyl serine is negatively charged. In the preferred embodiment the lipids have an overall negative charge. In other embodiments of this invention it is possible to use combinations of other lipids. A tissue factor:phospholipid molar ratio of about 1:2,000 to 1:20,000 is required with the most preferred ratio being about 1:10,000. This results in a PT reagent with a total phospholipid concentration of about 1-300 μM. A preferred source of the natural PS is from bovine brain and a preferred source of the natural PC is from egg yolk.

Synthetic phospholipids may also be used with the present invention. These synthetic compounds may be varied and may have variations in their fatty acid side chains not found in naturally occurring phospholipids. The side chain variations that result in PT reagent

improvement are unsaturated fatty acid side chains with C14, C16, or C18 chains length in either or both the PS or PC. Preferred compositions include but are not limited to those that have dioleoyl (18:1)-PS, palmitoyl (16:0)-oleoyl (18:1)-PS, dimyristoyl (14:0)-PS, dipalmitoleoyl (16:1)-PC, dipalmitoyl (16:0)-PC, dioleoyl (18:1)-PC, palmitoyl (16:0)-oleoyl (18:1)-PC, and myristoyl (14:0)-oleoyl (18:1)-PC as constituents.

Optimal activity of the PT reagent is achieved when the tissue factor:synthetic phospholipid ratios are about 1:2,000 to 1:20,000 with the preferred ratio being about 1:10,000. This leads to a final concentration of about 1-300 μM of total phospholipids. Thus both the PS:PC and rTF to toal phospholipid ratio are essential to achieve and maintain optimal functional activity.

The PT reagents made from recombinant or natural purified tissue factor in combination with natural phospholipids and synthetic phospholipids with and without variation in side chains offers an improvement in the quality and sensitivity of the PT reagent. Synthetic phospholipids give the advantage of a more reproducible final product and offer the improvement of better controlled functional activity of the PT reagent when the side chains are varied.

The choice of buffers and stabilizers vary widely and can also assist in the stability of the PT reagent. The most preferred embodiments may include calcium ion in the concentration range from about 9 to 15 mM, NaCl in the concentration range from about 0 to 10% with the most preferred range from about 2 to 5%, dextran in the range of about 0 to 5%, and an appropriate buffer. Buffers, such as N-2-Hydroxyethylpiperazine-N'-2-aminoethane sulfonic acid (HEPES), 3-[N-(Tris-

acid (TAPSO), 3-(N-Morpholino) propane sulfonic acid (MOPS), N-Tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), 3-[N-bis(hydroxyethyl)-amino]2-hydroxypropane sulfonic acid (DIPSO), Piperazine-N, N' bis (2-hydroxypropane-sulfonic acid) (POPSO), N-Hydroxyethylpiperazine-N'-2-hydroxypropane sulfonic (HEPPSO) and Tris-(hydroxymethyl) aminomethane (TRIS) are preferred in the PT reagent. The most preferred buffers are HEPES or TAPSO in the concentration ragne of about 20 to 80 mM.

In the preferred embodiment of this invention, the raw material recombinant human tissue factor is grown in vitro in E. coli, extracted with a detergent solution and then purified using affinity chromatography methods on immobilized monoclonal antibodies directed against human tissue factor. Bach, Ronald R., Initiation of Coagulation by Tissue Factor, CRC Critical Reviews in Biochemistry 1988; 23 (4):pp.339-368.

In the method of this invention, the purified tissue factor is combined with mixtures of either purified natural or specific synthetic phospholipids as previously described. This process is performed by mixing the recombinant protein in a detergent, such as octylglucoside or a similar detergent, with the phospholipids, also solubilized in a detergent solution. The detergents should have a critical micelle concentration high enough to allow diafiltration. The detergents are then removed by a diafiltration or dialysis process to form lipid micelles that contain the tissue factor.

The diafiltration is accomplished as follows: Phospholipids of this invention for example, phosphatidyl choline: phosphatidyl serine at about a rataio of about 70:30, either natural or synthetic are

solubilized by vortexing, mixing, heating and/or water bath sonication in a detergent with a critical micelle concentration high enough to allow its diafiltration. The phospholipids of the mixture are at aobut 8 to 20 mM and preferably at 10 mM. The critical micell concentration of the detergent preferably is greater than about 1 x 10^{-4} m/L with the most preferred concentration at about 2.5 x 10^{-2} m/L. For example, with octyglucoside or other similar detergents, the lipids preferable can be solubilized in a concentration range of detergent at about 11 mg/mL to 220 mg/mL, and most preferable at about 110 mg/mL. The lipid mixtures are combined at room temperature with rTF dissolved in a range from about .1 to 10% detergents and preferably at about 1% octylglucoside or other detergents. The other detergents of this invention may include non-ionic glucopuranosides, polyoxyethylene and non-denaturing zwitterionic detergents. The preferred detergent is octylglucoside. The lipid/rTF mixtures are immediately diluted about 1:1 with buffer and pumped in the vessel of a Membrex Benchmark^{R} GX Biofiltration System, or other tangential flow system, making contact with the exterior of the membrane. As detergent flows out through the pores of the membrane, buffer is pumped in. The sample is being re-circulated during this process and the biofiltration membrane is rotating to prevent lipid build-up at the surface and to force buffer through the filter. Alternatively, lipid build-up may be prevented by sweeping material tangential to the filter surface such as occurs with any tangential flow filtration device. Alternatively, the membrane is stationary as in the Membrex Pacesetter^{R} VFF System. Vortices that sweep the membrane are generated by the movement of a rotor that runs down the center f the

membrane. Alternatively, if the detergents used to dissolve the rTF and the phospholipids are the same detergent at the same concentration, then the rTF and the phospholipids may be added together.

After about 20-50 volumes of buffer, the detergent removal is complete and lipid micelles containing rTF have been formed. To ensure that detergent removal is complete, a PT assay is performed using normal and abnormal control plasmas. A prolongation in PT times and high ratios of abnormal to normal PT values indicate that residual detergent is still present. The sample is concentrated and assayed for functional activity. The diafiltration process is more efficient and reproducible than current processes which use dialysis. The diafiltration process requires much less volume and is less time consuming than the current dialysis processes that are employed. The detergent-free protein:phospholipid mixture is then added to a solution of buffers and stabilizers. The mixture is stirred to ensure homogeneity, dispensed into vials and then frozen and freeze-dried (lyophilized). The dried reagent is reconstituted to its active form by the addition of water.

PT values can be determined by any of the commonly used end point detection methods including mechanical and photo-optical instruments. The enhanced clarity of PT reagents based on this composition is particularly advantageous for photo-optical instruments.

The PT reagents of this invention show a improved stability before and after reconstitution over commercially available PT reagents. Table II shows the improvement in stability of PT values using a normal control tested with a reconstituted truncated rTF PT reagent compar d with a commercially available reagent.

COMPARISON OF RECONSTITUTED STABILITY

Temperature	PT Reagent of This Invention	PT Reagent Available Commercially
37°C	24 hours	8 hours
25°C	5 days	1 day
2-8°C	10 days	5 days

TABLE II

Figure 1 demonstrates this stability graphically. A lyophilized PT reagent of this invention which was prepared with a truncated rTF and a commercially available PT reagent were reconstituted and stored at 37°C. At various hours, a normal control was tested with both types of PT reagents. The PT values obtained were compared with PT values obtained for the same normal control using freshly reconstituted PT reagents of both types. The PT reagent of this invention shows an improved reconstituted stability over the commercially available PT reagent.

Figure 2 demonstrates the improvement in unreconstituted (dried) stability at 37°C of a truncated rTF lyophilized PT reagent when compared with a commercially available reagent. The data was obtained by storing several unreconstituted vials of each type of PT reagent at 37°C and reconstituting a fresh vial of both types of PT reagents on the indicated days. Vials stored at 2-8°C for both types of PT reagents were used as a control for both types of PT reagent. The normal control was tested on these days and PT values obtained using the vials stored at 37°C were compared to the PT values obtained for vials stored at 2-8°C at each day

for both types of PT reagents. The difference in PT values between vials stored at the two temperatures was calculated and the change was plotted against the days tested. The PT reagent of this invention shows an improved unreconstituted stability over the commercially available PT reagent.

EXAMPLE I

PT Reagents made using Full Length recombinant Human Tissue Factor and Natural Phospholipids - Effect of Varying rTF Concentration

Various concentrations of recombinant human tissue factor were lipidated with purified bovine phosphatidyl serine (PS) and purified egg phosphatidyl choline (PC) in a PS:PC ratio of 30:70 and a molar ratio of 1:10,000 rtf:phospholipid. The formulation also included 50 mM TAPSO, 11 mM $CaCl_2$, 2.6% glycine, 2.6% dextran, pH 7.4. Results are given as clotting times and were determined using an MLA Electra 800 photo-optical coagulation timer. Two commercial PT reagents based on rabbit brain tissue factor, Thromboplastin C+ and Thromboplastin IS, are included for comparison. The test plasmas are a normal lyophilized control, Ci-Trol 1 (COL 1), an abnormal lyophilized control, Ci-Trol II (COL 2), a pool of fresh normal plasma (FNP), and a lyophilized pool of plasmas from patients receiving warfarin (WARFARIN). The column under WAR/FNP is the ratio of the PT value of the warfarinized plasma pool divided by the PT value of the normal plasma pool. This ratio is a measure of sensitivity of the reagents. See Data Table I.

rTF Concentration (ng/mL)	COL1	COL2	FNP	WARFARIN	WAR/FNP
12.5	12.7	28.4	13.1	31.6	2.41
25.0	11.5	26.2	11.8	28.1	2.31
50.0	10.8	24.2	10.6	25.8	2.43
100.0	9.5	23.0	9.6	24.5	2.55
150.0	9.1	23.1	9.2	24.5	2.66
200.0	8.9	23.1	9.0	24.8	2.76
300.0	8.9	23.8	8.8	25.4	2.89
400.0	8.9	24.4	8.7	26.4	3.03
THROMBOPLASTIN C+	11.8	22.9	11.7	24.4	2.09
THROMBOPLASTIN IS	14.3	35.4	13.4	37.1	2.77

DATA TABLE I

EXAMPLE II

PT Reagents made using Full Length recombinant Human Tissue Factor and Natural Phospholipids - Effect of Varying rTF:Phospholipid Ratio (Lyophilized Reagents)

Recombinant human tissue factor, at either 145 ng/mL or 200 ng/mL, was combined with a mixture of purified bovine phosphatidyl serine (PS) and purified egg phosphatidyl choline (PC) in a PS:PC ratio of 30:70. In the example shown, two molar ratios of rTF:phospholipid, 1:10,000 and 1:20,000 rTF:phospholipid, were used. The first formulation (10S, 20S) with 145 ng/mL rTF, also included 68 mM TAPSO, 11 mM $CaCl_2$, 140 mM NaCl, 5.2% glycine, pH 7.4. The formulations were dispensed into vials and freeze-dried. Results are given as clotting times and were determined using an MLA Electra 700 photo-optical coagulation timer. A commercial PT reagent, Thromboplastin IS, is included for comparison. The test plasmas are a normal lyophilized control, Ci-Trol 1 (COL q), an abnormal lyophilized control, Ci-Trol II (COL 2), a pool of fresh normal plasma (FNP), and a lyophilized

pool of plasmas from patients receiving warfarin (WARFARIN). The column under WAR/FNP is the ratio of the PT value of the warfarinized plasma pool divided by the PT value of the normal plasma pool. This ratio is a measure of sensitivity of the reagents. See Data Table 2.

FORMULATION rTF:LIPID	COL1	COL2	FNP	WARFARIN	WAR/FNP
10S (1:10,000)	11.0	28.5	9.9	37.1	3.75
20S (1:20,000)	11.8	30.8	10.6	40.1	3.78
10F (1:10,000)	11.0	28.7	9.9	39.2	3.96
20F (1:20,000)	12.1	31.9	10.8	44.5	4.12
THROMBOPLASTIN IS	15.0	40.9	14.0	45.3	3.24

DATA TABLE 2

EXAMPLE 3

PT Reagents made using Full Length recombinant Human Tissue Factor and Synthetic Phospholipids - Effect of Varying the Nature of the Fatty Acid Side Chain Moiety of the Phospholipid

Purified recombinant human tissue factor, at eigher 100 or 300 ng/mL, was combined with mixtures of synthetic phosphatidyl serine (PS) and synthetic phosphatidyl choline (PC) in a PS:PC ratio of 30:70 and a ratio of rTF:phospholipid of 1:10,000. The formulation also included 50 mM TAPSO, 11 mM $CaCl_2$, 100 mM NaCl, pH 7.4. Results are given as clotting times and were determined using an MLA Electra 800 photo-optical coagulation timer. Recombinant tissue factor lipidaced with natural phospholipids, bovine PS and egg PC, was used as a control. The test plasmas are a normal lyophilized control, CiTrol 1 (COL 1), an

abnormal lyophilized control, CiTrol II (COL 2), and a lyophilized pool of plasmas from patients receiving warfarin (WARFARIN). The column under RAT is the ratio of the ratio of the PT value of the warfarinized plasma pool divided by the PT value of COL 1. This ratio is a measure of sensitivity of the reagents. See Data Table 3.

PS Description	PC Description	RTF ng/mL	COL1	COL2	WARF	RAT
Dimyristoyl(14:0)	Dilauroyl(12:0)	100	24.3	54.2	53.1	2.19
		300	19.1	46.6	44.6	2.34
Dimyristoyl(14:0)	Dimyristoyl(14:0)	100	40.4	>100	82.7	2.05
		300	41.1	>100	100	2.43
Dimyristoyl(14:0)	Dipalmitoyl(16:0)	100	96.3	>100	>200	
		300	93.2	>100	>200	
Dimyristoyl(14:0)	Dipalmitoleoyl (16:0)	100	16.9	38.4	35.8	2.12
		300	14.1	33.5	29.7	2.11
Palm(16:0)-oleoyl (18:1)	Dimyrstoyl(14:0)	100	17.7	38.3	34.7	1.96
		300	16.4	37.8	35.6	2.17
Dioleoyl(18:1)	Dipalmitoleoyl (16:0)	100	10.2	23.1	19.3	1.89
		300	10.0	22.6	19.2	1.92
Palm(16:0)-oleoyl (18:1)	Dipalmitoleoyl	100	11.8	24.5	21.1	1.79
		300	10.2	24.1	19.4	1.90
Dimyristoyl(14:0)	Dioleoyl(18:1)	100	11.2	26.5	22.7	2.03
		300	10.4	26.7	22.4	2.15
Palm(16:0)-oleoyl (18:1)	Dioleoyl(18:1)	100	10.3	24.4	19.2	1.86
		300	9.5	25.4	20.0	2.11
Dioleoyl(18:1)	Dipalmitoyl(16:0)	100	12.8	29.5	23.8	1.86
		300	11.0	27.8	21.8	1.98

PS Description	PC Description	RTF ng/mL	COL1	COL2	WARF	RAT
Palm(16:0)-oleoyl (18:1)	Dioleoyl(18:1)	100	11.3	26.9	20.6	1.82
		300	9.9	26.8	20.0	2.02
Palm(16:0)-oleoyl (18:1)	Palm(16:0)-oleoyl (18:1)	100	12.4	27.2	22.3	1.80
		300	10.5	24.4	19.7	1.88
Dioleoyl(18:1)	Palm(16:0)-oleoyl (18:1)	100	12.9	30.8	24.0	1.86
		300	10.6	29.5	21.8	2.06
Palm(16:0)-oleoyl (18:1)	Myr(14:0)-oleoyl (18:1)	100	13.4	29.3	23.1	1.72
		300	9.9	27.0	20.6	2.08
Dioleoyl(18:1)	Dioleoyl(18:1)	100	11.2	26.1	20.8	1.86
		300	11.9	26.7	21.1	1.77
Bovine	Egg	100	12.8	27.6	23.4	1.83
		300	10.5	22.9	19.4	1.85

DATA TABLE 3

EXAMPLE 4

PT Reagents made using Full Length recombinant Human Tissue Factor and Synthetic Phospholipids - Effect of Varying the Nature of the Fatty Acid Side Chain Moiety of the Phospholipid - Lyophilized Reagent

Purified recombinant human tissue factor, at 300 ng/mL, was combined with mixtures of synthetic phosphatidyl serine (PS) and synthetic phosphatidyl choline (PC) in a PS:PC ratio of 30:70 and a ratio of rTF:phospholipid of 1:10,000. The formulation also included 30 mM TAPSO, 11 mM $CaCl_2$, 215 mM NaCl, 3% glycine, pH 7.4. The mixtures were dispensed into vials and freeze-dried. Results are given as clotting times and were determined using an MLA Electra 800 photo-optical coagulation timer. The test plasmas are a

normal lyophilized control, Ci-Trol 1 (COL 1), an abnormal lyophilized control, Ci-Trol II (COL 2), a pool of fresh normal plasmas (FNP) and a lyophilized pool of plasmas from patients receiving warfarin (WARFARIN). The column under RAT is the ratio of the PT value of the warfarinized plasma pool divided by the PT value of the normal plasma pool. This ratio is a measure of sensitivity of the reagents. Two controls were included in the testing. The 10F control, lipidated with the natural phospholipids bovine PS and egg PC, was the 10F formulation given in Example II. The other control, Thromboplastin IS, is a commercially available high sensitivity rabbit brain-based thromboplastin reagent, Thromboplastin IS. See Data Table 4.

PS Description	PC Description	COL1	COL2	FNP	WARF	RAT
Dioleoyl(18:1)	Dipalmitoleoyl (16:1)	13.0	33.2	11.6	30.1	2.59
Palm(16:0)-oleoyl (18:1)	Dipalmitoleoyl	13.4	33.7	11.9	30.1	2.53
Dimyristoyl(14:0)	Dioleoyl(18:1)	19.4	54.2	16.9	53.0	3.14
Dioleoyl(18:1)	Dipalmitoyl(16:0)	17.1	43.7	15.6	37.7	2.42
Palm(16:0)-oleoyl (16:0)	Dioleoyl(18:1)	12.6	33.2	11.5	29.5	2.57
Palm(16:0)-oleoyl (18:1)	Palm(16:0)-oleoyl (18:1)	12.8	33.1	11.8	30.0	2.54
Dioleoyl(18:1)	Palm(16:0)-oleoyl (18:1)	13.0	35.2	11.8	31.2	2.64
Palm(16:0)-oleoyl (18:1)	Myris(14:0)-oleoyl (18:1)	11.8	28.6	11.3	24.2	2.14
Dioleoyl(18:1)	Dioleoyl(18:1)	12.3	33.6	11.0	31.0	2.82
Bovine Brain	Egg	13.5	35.9	12.1	34.2	2.83
10F		13.3	38.2	11.7	35.4	3.03
THROMBOPLASTIN-IS		14.2	37.6	13.5	27.4	2.03

DATA TABLE 4

EXAMPLE 5

PT reagents made using Truncated recombinant Human Tissue Factor and Synthetic Phospholipids - Effect of Varying the Nature of the Fatty Acid Side Chain Moiety of the Phospholipid - Lyophilized Reagents

Purified recombinant human tissue factor, containing 243 residues and missing most of the cytoplasmic portion of the molecule, was combined with mixtures of synthetic phosphatidyl serine (PS) and synthetic phosphatidyl choline (PC) in a PS:PC ratio of 30:70 and a ratio of rTF:phospholipid of 1:10,000. The formulation included 300 ng/mL rTF, 30 mM TAPSO, 11 mM $CaCl_2$, 215 M NaCl, 3% glycine, pH 7.4. Mixtures were dispensed into vials and freeze-dried. Results are given as clotting times and were determined using an MLA Electra 800 photo-optical coagulation timer. The test plasmas are a normal lyophilized control, Ci-Trol 1 (COL 1), an abnormal lyophilized control, Ci-Trol II (COL 2), a pool of fresh normal plasmas (FNP) and a lyophilized pool of plasmas from patients receiving warfarin (WARFARIN). The column under Ratio is the ratio of the PT value of the warfarinized plasma pool divided by the PT value of the normal plasma pool. This ratio is a measure of sensitivity of the reagents. Three controls were included in the testing, one was full length rTF as in previous examples, a second was truncated rTF lipidated with natural phospholipids, bovine PS and egg PC, and the third is a commercially available high sensitivity rabbit brain-based thromboplastin reagent, Thromboplastin IS. See Data Table 5.

PS Description	PC Description	COL1	COL2	FNP	WARF	RAT
TRUNCATED(342AA)rTF						
Bovine Brain	Egg	12.0	34.4	11.3	33.6	2.97
Dioleoyl(18:1)	Dioleoyl(18:1)	11.7	34.2	11.0	37.1	3.37
Palm(16:0)-oleoyl (18:1)	Dioleoyl(18:1)	12.1	33.9	11.6	36.1	3.11
Palm(16:0)-oleoyl (18:1)	Palm(16:0)-oleoyl (18:1)	12.3	34.0	11.9	34.7	2.92
Dioleoyl(18:1)	Palm(16:0)-oleoyl (18:1)	11.6	31.0	11.1	31.9	2.87
Palm(16:0)-oleoyl (18:1)	Myris(14:0)-oleoyl (18:1)	12.7	36.6	12.4	36.3	2.93
FULL LENGTH rTF						
Bovine Brain	Egg	12.6	33.4	12.5	34.4	2.75
Thromboplastin-IS		13.2	32.5	13.5	26.9	1.99

DATA TABLE 5

EXAMPLE 6

PT Reagents made using Truncated recombinant Human Tissue Factor and Synthetic Phospholipids - Effect of Varying the Concentration of rTF and the Reagent Composition

Purified recombinant human tissue factor, containing 243 residues and missing most of the cytoplasmic portion of the molecule, was combined with 30:70 mixtures of synthetic phospholipids at a rTF:phospholipid ratio of 1:10,000. Formulation A included synthetic 1-palmitoyl-2-oleoyl phosphatidyl serine (POPS) and dioleoyl phosphatidyl choline (DOPC), 60 mM HEPES, 11 mM $CaCl_2$, 200 mM NaCl, 4.6% glycine, 5 mg/L polybrene, pH 7.4. Formulation B included the same POPS and DPOC concentrations, 60 mM HEPES, 11 mM $CaCl_2$, 215 mM NaCl, 4.6% glycine, 5 mg/L polybrene, pH 7.4. Formulation C included dioleoyl phosphatidyl serine (DOPS) and DOPC, 40 mM TAPSO, 11 mM $CaCl_2$, 220 mM NaCl, 2.1% glycine, pH 7.4. mixtures were dispensed in vials and freeze-dried. Results are given as clotting times

and were determined using an MLA Electra 800 photo-optical coagulation timer. The test plasmas are a normal lyophilized control, Ci-Trol 1 (COL 1), an abnormal lyophilized control, Ci-Trol II (COL 2), a pool of fresh normal plasmas (FNP) and a lyophilized pool of plasmas from patients receiving warfarin (WARFARIN). The column under RATIO is the ratio of the PT value of the warfarinized plasma pool divided by the PT value of the normal plasma pool. This ratio is a measure of sensitivity of the reagents. A commercially available high sensitivity rabbit brain-based thromboplastin reagent, Thromboplastin IS, is included as a control. See Data Table 6.

FORMULATION	rTF Conc. (ng/mL)	COL1	COL2	FNP	WARFARIN	RATIO
A	100	14.2	32.1	14.8	35.2	2.38
	150	13.3	31.5	13.8	33.2	2.41
	180	12.9	31.4	13.6	33.0	2.43
	200	12.7	33.2	13.3	32.9	2.47
	240	12.7	32.3	13.3	32.9	2.47
	260	12.4	33.5	13.1	33.1	2.53
A	220	11.9	29.0	12.5	31.0	2.48
B	240	13.0	33.3	13.7	34.5	2.52
C	165	13.3	29.2	13.9	33.5	2.41
THROMBOPLASTIN-IS		14.0	35.3	15.5	29.2	1.88

DATA TABLE 6

EXAMPLE 7

PT Reagents made using Truncated recombinant Human Tissue Factor and Synthetic Phospholipids - Effect of Varying the POPS:DOPC Ratio and the Reagent Composition

Purified recombinant human tissue factor, containing 243 residues and missing most of the cytoplasmic portion of the molecule, was combined with

varyi mixtures of synthetic 1-palmitoyl-2-oleoyl phosphatidyl serine (POPS) and dioleoyl phosphatidyl choline (DOPC) at a ratio of rTF:phospholipid of 1:10,000. Formulation A included 220 ng/mL rTF with different ratios of POPS:DOPC, 60 mM HEPES, 11 mM $CaCl_2$, 215 mM NaCl, 4.6% glycine, 5 mg/L polybrene, pH 7.4. Formulation B included 240 ng/mL rTF with 30:70 POPS:DOPC, 60 mM HEPES, 11 mM $CaCl_2$, 215 mM NaCl, 4.6% glycine, pH 7.4. Formulation C included 165 ng/mL rTF with 30:70 DOPS:DOPC, 40 mM TAPSO, 11 mM $CaCl_2$, 220 mM NaCl, 2.1% glycine, pH 7.4. Mixtures were dispensed into vials and freeze dried. Results are given as clotting times and were determined using an MLA Electra 800 photo-optical coagulation timer. The test plasmas are a normal lyophilized control, Ci-Trol I (COL 1), an abnormal lyophilized ocntrol, Ci-Trol II (COL 2), a pool of fresh normal plasmas (FNP) and a lyophilized pool of plasmas from patients receiving warfarin (WARFARIN). The column under RATIO is the ratio of the PT value of the warfarinized plasma pool divided by the PT value of the normal plasma pool. This ratio is a measure of sensitivity of the reagents. A commercially available high sensitivity rabbit brain-based thromboplastin reagent, Thromboplastin IS, is included as a control. See Data Table 7.

FORMULATION	PS:PC	COL1	COL2	FNP	WARFARIN	RATIO
A	25:75	12.4	31.1	13.7	33.3	2.43
	30:70	11.5	28.2	12.5	30.4	2.43
	35:65	11.1	24.8	12.0	26.7	2.43
A	30:70	11.6	27.7	12.5	30.3	2.42
B	30:70	12.6	31.4	13.4	34.4	2.57
C	30:70	12.0	26.8	13.1	34.0	2.60
THROMBOPLASTIN-IS		14.1	35.5	15.2	29.8	1.96

DATA TABLE 7

WE CLAIM:

1. A prothrombin time reagent comprising:
   (a) a recombinant protein having substantially the amino acid sequence of human tissue factor;
   (b) a phospholipid in an amount sufficient to activate said protein;
   (c) a buffer composition; and
   (d) calcium ion in an amount sufficient to activate the recombinant protein.

2. A prothrombin time reagent comprising:
   (a) a recombinant protein having an amino acid sequence corresponding substantially to the cytoplasmic portion of human tissue factor;
   (b) a phospholipid in an amount sufficient to activate said protein;
   (c) a buffer composition; and
   (d) calcium ion in an amount sufficient to activate the recombinant protein.

3. A prothrombin time reagent comprising:
   (a) a recombinant protein having substantially the amino acid sequence of human tissue factor;
   (b) a mixture of phospholipids in an amount sufficient to activate said protein;
   (c) a buffer composition; and
   (d) calcium ion in an amount sufficient to activate the recombinant protein.

4. The prothrombin time reagent of claim 1 or 2 wherein the phospholipid is selected from the group consisting of phosphatidyl choline and phosphatidyl serine.

5. The prothrombin time reagent of claim 3 wherein the mixture of phospholipids is selected from the group consisting of phosphatidyl choline and phosphatidyl serine.

6. The prothrombin time reagent of claims 1 and 2 wherein the phospholipid is synthetic.

7. A method for preparing lipid micelles containing tissue factor comprising:

(a) solubilizing the phospholipids in a detergent;

(b) combining tissue factor with the solubilized phospholipids;

(c) placing the combined phospholipid and tissue factor mixture in a tangential flow system in such a way that said mixture makes contact with the membrane of said system; and

(d) flushing the system with buffer.

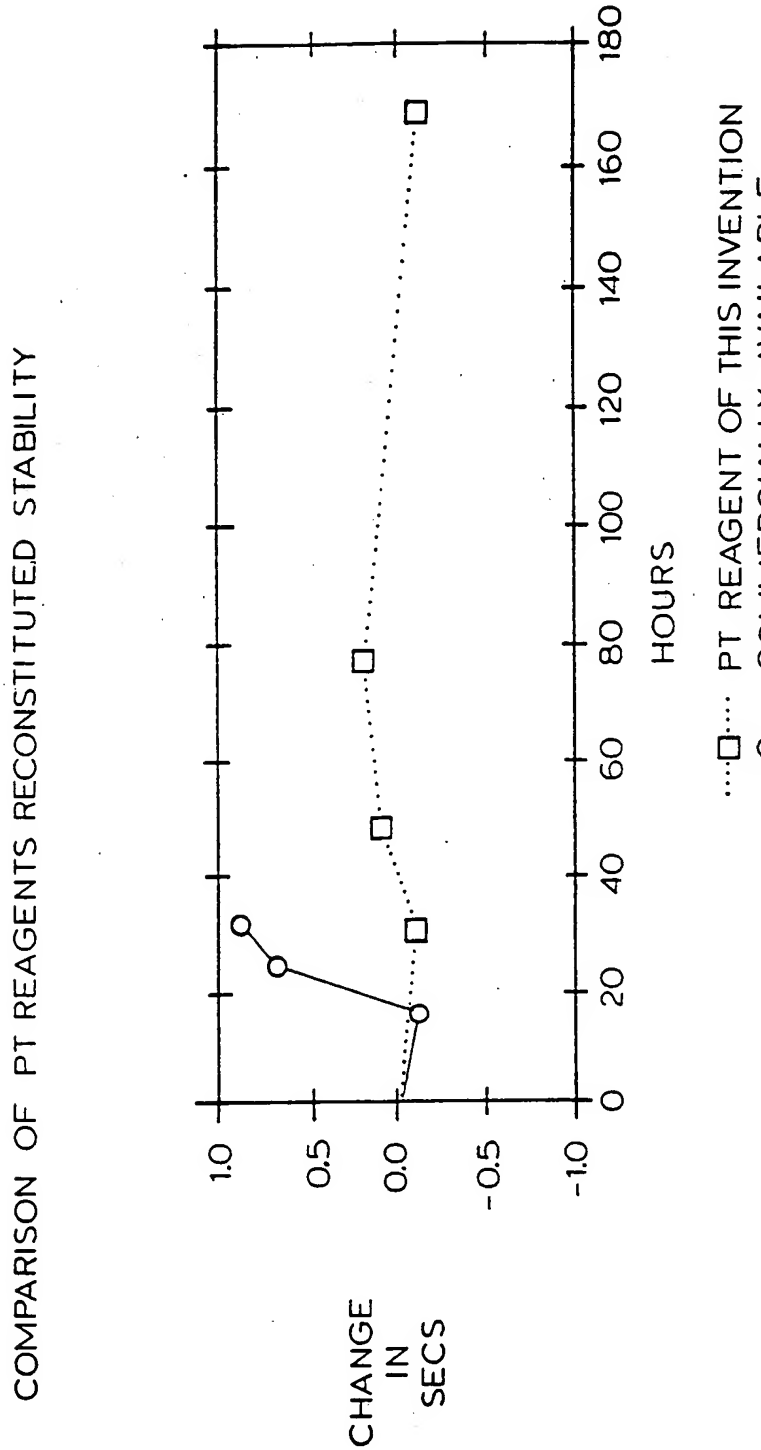

COMPARISON OF PT REAGENTS RECONSTITUTED STABILITY
CHANGE IN SECS
1.0
0.5
0.0
-0.5
-1.0
0
20
40
60
80
100
120
140
160
180
HOURS
PT REAGENT OF THIS INVENTION
COMMERCIALLY AVAILABLE PT REAGENT

FIG. 1

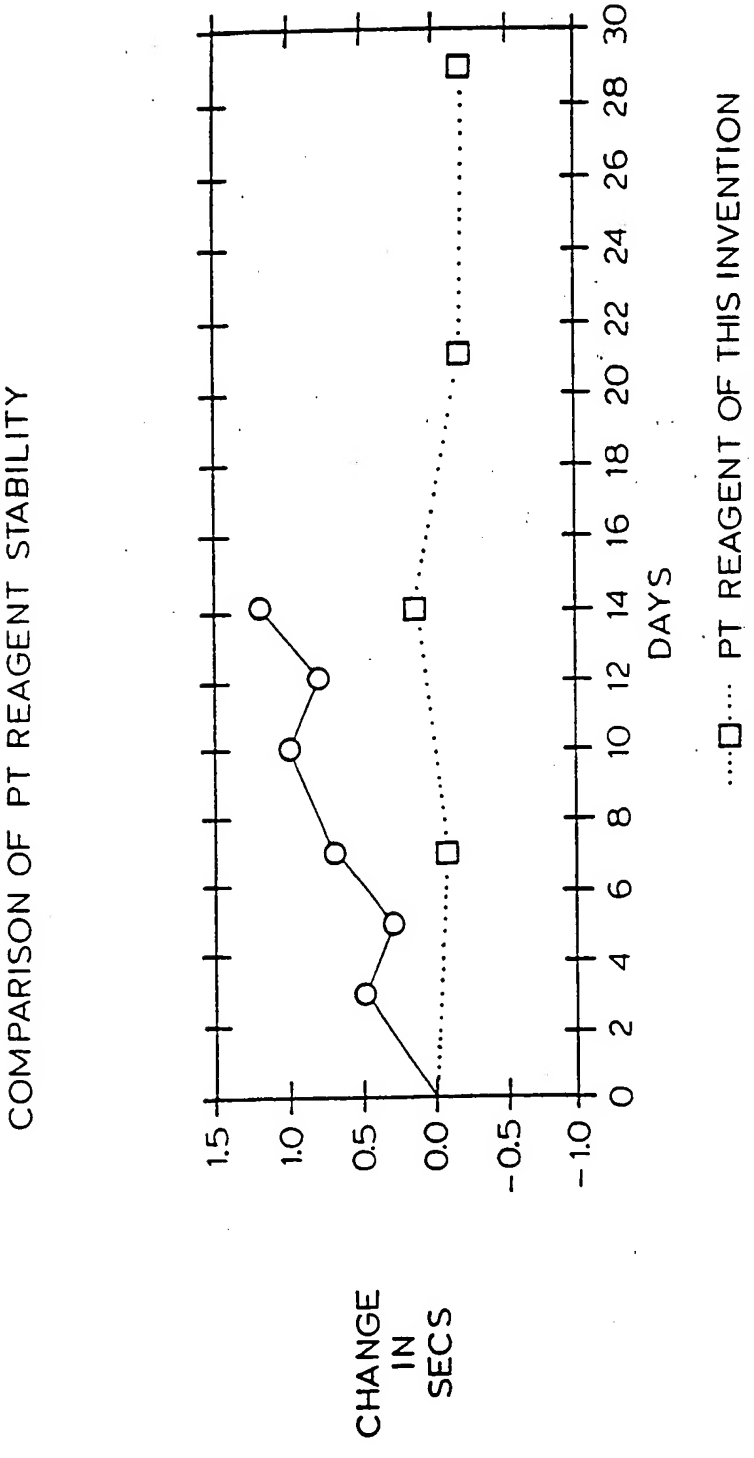

COMPARISON OF PT REAGENT STABILITY
CHANGE IN SECS
1.5
1.0
0.5
0.0
-0.5
-1.0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
DAYS
PT REAGENT OF THIS INVENTION
COMMERCIALLY AVAILABLE PT REAGENT

FIG. 2

INTERNATIONAL SEARCH REPORT

PCT/US 92/08281

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/86; //A61K9/127

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	EP,A,0 014 039 (ORTHO DIAGNOSTICS INC.) 6 August 1980 see page 1 - page 6, line 4 ---	1-6
Y	BLOOD vol. 71, no. 1, January 1988, ORLANDO, USA pages 1 - 8 Y. NEMERSON 'Tissue factor and hemostasis' cited in the application see the whole document --- -/--	1-7

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 JANUARY 1993	-2. 02. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	GRIFFITH G.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 84, August 1987, WASHINGTON US pages 5148 - 5152 E. K. SPICER ET AL. 'Isolation of cDNA clones coding for human tissue factor: Primary structure of the protein and cDNA' cited in the application see page 5148	1-7
Y	BIOCHEMISTRY vol. 28, no. 20, 1989, EASTON, PA US pages 8072 - 8077 'l. p. paborsky et al.' cited in the application see page 8072 - page 8073	1-7
Y	BIOCHEMISTRY vol. 25, no. 14, 1986, EASTON, PA US pages 4077 - 4020 R. BACH 'Factor VII bindingto tissue factor in reconstituted phospholipid vesicles: Induction of cooperativity by phosphatatidylserine' see the whole document	1-6 7
X		
P,X	WO,A,9 208 479 (CORVAS INTERNATIONAL, INC.) 29 May 1992 see the whole document	1-7
E	WO,A,9 218 870 (OKLAHOMA MEDICAL RESEARCH FOUNDATION) 29 October 1992 see the whole document	1-7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9208281
SA 65540

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 22/01/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0014039	06-08-80	US-A- 4289498	15-09-81
		CA-A- 1136029	23-11-82
		JP-A- 55124071	24-09-80
WO-A-9208479	29-05-92	AU-A- 9090791	11-06-92
WO-A-9218870	29-10-92	None	

EP FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82